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EXHIBIT A

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Calpain inhibitors and antioxidants act synergistically to prevent cell necrosis: effects of the novel dual inhibitors (cysteine protease inhibitor and antioxidant) BN 82204 and its pro-drug BN 82270

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Abstract

Cell death is a common feature observed in neurodegenerative disorders, and is often associated with calpain activation and overproduction of reactive oxygen species (ROS). This study investigated the use of calpain inhibitors and antioxidants in combination to protect cells against necrosis. Maitotoxin (MTX), which induces a massive influx of calcium, was used to provoke neuronal cell death. This toxin increased, in a concentration-dependent manner, both calpain activity and ROS formation. Calpain inhibitors or antioxidants inhibited MTX-induced necrosis only marginally (below 20%), whereas their association protected against cell death by 40–66% in a synergistic manner. BN 82204, which possesses both calpain–cathepsin L inhibitory and antioxidant properties, and its acetylated pro-drug BN 82270, totally protected cells at 100 μM. The pro-drug BN 82270, which had better cell penetration, was twice as effective as the active principle BN 82204 in protecting glioma C6 or neuroblastoma SHSY5Y cells against death. These results suggest the potential therapeutic relevance of using a single molecule with multiple activities (cysteine protease inhibitor/antioxidant), and warrant further *in vivo* investigations in models of neuronal disorders.

Keywords: antioxidant, C6 and SHSY5Y cells, calpain, cathepsin, maitotoxin, synergistic protection.

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Various mechanisms have been implicated in neurodegenerative disorders, including an increase in calpain activity and the release of free radicals.

A large body of evidence has linked neuronal death to free radicals and oxidative damage (Coyle and Puttfarcken 1993; Beal 1996; Halliwell 2001; Jenner 1998; Owen *et al.* 1996; Simonian and Coyle 1996). Free radicals in tissues are generated both enzymatically and non-enzymatically, leading to the formation of reactive oxygen species (ROS). The incorporation of molecular oxygen into polyunsaturated fatty acids initiates a chain of reactions in which ROS, including hydroxyl radicals, hydrogen peroxide, peroxyl and alkyoxyl radicals, are formed (Halliwell *et al.* 1985). ROS, especially hydroxyl radicals, can produce functional alterations in lipids, proteins and nucleic acids. Oxidative lipid damage, termed lipid peroxidation, produces a progressive loss of membrane fluidity, reduces membrane potential and increases permeability to ions such as calcium. Finally, antioxidants have been shown to afford significant protection in disorders involving necrosis (Bittigau *et al.* 1998; Naoi *et al.* 1998; Casetta *et al.* 2005; Hashizume *et al.* 2005).

Calpain is a Ca^{2+}-activated cysteine protease typically associated with cellular necrosis (Lipton 1999). A substantial increase in the proteolytic activity of calpain, probably due to loss of ionic homeostasis and a rise in intracellular calcium concentration, occur after traumatic (Kampfl *et al.* 1996, 1997) or ischaemic (Bartus *et al.* 1994a, 1994b, 1995;

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Abbreviations used: AC-DEVD, Acetyl-Asp-Glu-Val-Asp; AMC, 7-amino-4-methylcoumarin; BHT, 3,5-di-*ter*butyl-4-hydroxybenzoic acid; Cath. L Inh., cathepsin L inhibitor IV; Cbz=Z, Benzyloxycarbonyl; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EIA, Enzyme immunoassay; F2-IP, F2-isoprostane; FCS, fetal calf serum; H, aldehyde; Inh., inhibitor; 8-iso PGF2α, prostaglandin F2 alpha; MDA, malonaldhehyde; MTX, maitotoxin; NF, neurofilament; NFP, neurofilament protein; ns, not significant; NSF, no-survival fraction; ROS, reactive oxygen species; SF, survival fraction; Suc, succinyl; WST-1, tetrazolium salt (4-[3-(4-iodophenyl)2–2(4-nitrophenyl)2H-5-tetrazolio]-1,3-benzene disulphonate).

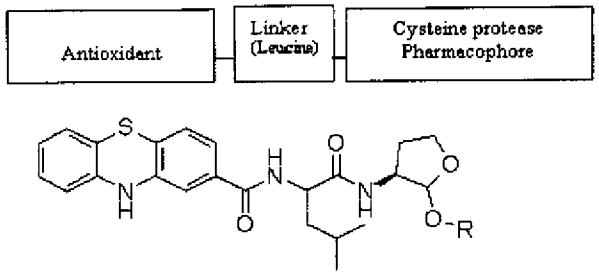
EXHIBIT A

Roberts-Lewis *et al.* 1994) brain injury, as well as in spinal cord injury (Banik *et al.* 1987, 1997, 1998; Li *et al.* 1995; Springer *et al.* 1997; Schumacher *et al.* 1999). Calpain in turn acts on several substrates, many of which are cytoskeletal proteins (Saido *et al.* 1994). Cytoskeletal degradation may feature prominently in the commitment of an injured cell to necrotic death. Hence, pharmacological inhibition of calpain should lead to significant sparing of cells. Indeed, it has been shown that intravenous infusion of the calpain inhibitor calpeptin in a rat model of spinal cord injury inhibited degradation of neurofilament (NF)68 and NF200, reduced cell death (Banik *et al.* 1998), and provided significant neuroprotection (Ray *et al.* 2003).

Calpain, stimulated by free radicals, also mediates apoptotic cell death. Banik *et al.* (1998) suggested that the use of calpain and lipid peroxidation drugs as therapeutic agents would protect cells and maintain the axon–myelin structural unit by preventing protein degradation. Banik and colleagues found that combination treatment with calpeptin and methylprednisolone used as free-radical inhibitor was slightly more effective in preventing neurofilament protein (NFP) degradation, compared with either agent used alone (Banik *et al.* 1998).

Maitotoxin (MTX) was first discovered as one of the toxins responsible for ciguatera, a seafood poisoning caused by ingestion of coral reef fish, and was later shown to be a metabolite of the dinoflagellate *Gambierdiscus toxicus*. MTX at 0.3 nM elicited a 10–20-fold increase in the level of Ca^{2+} in rat glioma C6 cells (Konoki *et al.* 1999). In fact, MTX elicits Ca^{2+} influx in virtually all cells and tissues (Escobar *et al.* 1998). It activates both voltage-sensitive (Freedman *et al.* 1984) and receptor-operated (Gusovsky *et al.* 1993) calcium channels in the plasma membrane. This results in calcium overload that rapidly leads to cell death. Zhao *et al.* (1999) demonstrated that MTX induces calpain activation and necrotic cell death in primary septohippocampal cultures. Their results suggest that: (i) calpain is activated as a result of MTX-induced Ca^{2+} influx; (ii) necrotic cell death caused by MTX exposure is partially mediated by calpain activation; and (iii) MTX is a useful tool with which to investigate pathological mechanisms of necrosis.

The first aim of this study was to set up a cellular model of cell death, involving calpain activation and free radical production, mimicking pathological cell death conditions. The second aim was to determine the effect of simultaneous treatment with a calpain inhibitor and a free radical scavenger. Various associations of the two compounds were performed to quantify the protective effect of co-treatment (antagonistic, additive, synergistic). The final aim was to measure the neuroprotective effect of a single synthetic molecule, BN 82204, and its acetylated pro-drug, BN 82270, (Fig. 1) on two neuronal cell types (C6 astroglioma and SHSY5Y neuroblastoma).



R= $COCH_3$; masked calpain pharmacophore : pro-drug BN 82270

cleavage

R = H; BN 82204 active principle

Fig. 1 Chemical structure of BN 82204 and BN 82270. The antioxidant moiety (phenothiazine) is linked to the calpain inhibitor pharmacophore, 2-hydroxytetrahydrofuran, by a leucine residue. In BN 82270, the calpain pharmacophore is masked by an acetyl group. BN 82270 is devoid of effect *per se* on isolated human calpain enzyme, and needs to penetrate the cell where the acetyl group is cleaved, leading to the active principle that inhibits calpain (Auvin *et al.* 2004).

Our results demonstrate that calpain activation and free radical overproduction resulting from MTX treatment is associated with cell death. Furthermore, the combination of a calpain inhibitor and an antioxidant protected against cell death by 40–66% in a synergistic manner. BN 82204, which was chemically designed as a dual calpain and free radical inhibitor, and its pro-drug BN 82270 also protected cells. Total cell protection was observed with these synthetic molecules, in contrast to the limited protection obtained with the combination of a calpain inhibitor and an antioxidant. This may be attributed to the inhibition of cathepsin L, an additional property possessed by these compounds. These results suggest that such multifunctional products could be useful as therapeutic agents in neurodegenerative disorders in which massive influx of calcium is implicated.

Materials and methods

Products and reagents

BN 82204 and BN 82270 [(3*S*)-3-{[*N*-(10H-phenothiazin-2-ylcarbonyl) leucyl]amino} tetrahydrofuran-2-yl –/+acetate] are shown in Fig. 1. Note that BN 82270, the pro-drug of BN 82204, has a calpain pharmacophore (2-hydroxytetrahydrofuran group) protected by an acetyl group that is removable under biological conditions. The synthesis of both compounds has already been described Auvin *et al.* (2004).

BN compounds were dissolved in dimethylsulfoxide (DMSO) (2×10^{-2} M). Stock solutions were freshly made for each experiment. Reference compounds, antioxidants, calpain inhibitors and cathepsin inhibitors were dissolved in DMSO (2×10^{-2} M). Aliquots were stored at −80°C. Dulbecco's modified Eagle's medium

(DMEM) and fetal calf serum (FCS) were from Gibco-BRL (Fisher Bioblock Scientific, Illkirch, France), and 96-well plates were from Costar (Corning, Avon, France). Succinyl-Leu-Tyr-7-amido-4-methylcoumarin (Suc-Leu-Tyr-AMC), Cbz-Phe-Arg-AMC (Z = Benzyloxycarbonyl) and Suc-Ala-Ala-Pro-Phe-AMC were from Bachem (Bubendorf, Switzerland). Acetyl-Asp-Glu-Val-Asp (AC-DEVD-AMC), Suc-Leu-Leu-Val-Tyr-AMC, human calpain 1, porcine calpain 2, human cathepsin L, human recombinant caspase 3, rabbit 20S proteasome, calpeptin, 3-(4-iodophenyl)-2-mercapto-2-propenoic acid (PD150606), α-chymotrypsin inhibitor I, cathepsin L inhibitor IV, caspase 3 inhibitor I and MG132 were from Calbiochem (San Diego, CA, USA). Chromogenic reagent *N*-methyl-2-phenylindole was from Sigma-Aldrich (St Louis, MO, USA). MTX was from Wako (Osaka, Japan). Benzyloxycarbonyl (Z)-Leu-Leu-aldehyde (H) was from Biomol (le Perray en Yvelines, France), Z-Leu-Phe-H, 4-hydroxydiphenylamine and 2-methoxyphenothiazine were prepared by Ipsen (Les Ulis, France). 3,5-Di-*ter*butyl-4-hydroxybenzoic acid (BHT) was from Sigma (St Louis, MO, USA). The Enzyme immunoassay (EIA) kit for 8-*iso* Prostaglandin F2 alpha (8-*iso* PGF2)α was from Cayman chemical. Cell proliferation reagent tetrazolium salt (4-[3-(4-iodophenyl)2-2(4-nitrophenyl)2H-5-tetrazolio]-1,3-benzene disulphonate; WST-1) was from Roche (Indianapolis, IN, USA). All other chemicals used were of the highest quality commercially available.

Enzymatic assay of human calpain 1 activity

This fluorogenic assay was performed in a 96-well plate (Tompa *et al.* 1995). Ten microlitres of compound was mixed with 45 μL buffer containing human calpain 1 (at a final concentration of 1 U/mL) and Suc-Leu-Tyr-AMC. The dipeptide Suc-Leu-Tyr-AMC was used at a final concentration of 0.5 mM in buffer containing 110 mM Tris-HCl, 110 mM NaCl, 2.2 mM EDTA, 2.2 mM EGTA and 1.1 mM mercaptoethanol. The reaction was initiated by addition of 45 μL $CaCl_2$ at a final concentration of 10 mM. Proteolysis of the fluorescent peptide was read using a fluorescent plate-reading system (Victor Wallac, PerkinElmer, Courtaboeuf, France) at 380 nm excitation and 460 nm emission. The results were expressed as the concentration that inhibits 50% of human calpain 1 activity (IC_{50}).

Assays for cysteine, aspartyl and serine proteases and proteasome activities

All fluorogenic assays were performed in 96-well plates. The assays used were based on the ability of the enzymes to cleave synthetic peptidic fluorescent substrates. Suc-Leu-Tyr-AMC (0.5 mM) was used with porcine calpain 2 (2.5 U/mL) in 500 mM Tris-HCl buffer, pH 7.5, containing 1 M NaCl, 1 mM EDTA, 1 mM EGTA and 0.5 mM mercaptoethanol. The reaction was initiated by addition of 45 μL $CaCl_2$ at 10 mM. Cbz-Phe-Arg-AMC (300 μM) was used with human cathepsin L (0.1 U/mL) in 20 mM Na-O-acetate by sodium acetate buffer, pH 6, containing 1 mM EDTA and 1 mM dithiothreitol. Suc-Ala-Ala-Pro-Phe-AMC (550 μM) was used with bovine α-chymotrypsin (0.055 U/mL) in 50 mM Tris-HCl buffer, pH 7.5, containing 500 mM NaCl. AC-DEDV-AMC (50 μM) was used with human caspase 3 (100 U/mL) in 25 mM HEPES buffer, pH 7.5, containing 1 mM EDTA, 2 mM dithiothreitol, 0.1% CHAPS and 10% sucrose. Suc-Leu-Leu-Val-Tyr-AMC (10 μM) was used with rabbit 20S proteasome (0.25 nM) in 20 mM HEPES buffer, pH 7.8, containing 0.5 mM EDTA and 0.035% sodium dodecyl sulphate. The fluorescence of the cleavage product 4-methylcoumarin (AMC) was monitored using a fluorescent plate-reading system (excitation at 380 nm and emission at 460 nm). The amount of AMC fragment formed was directly related to the isolated enzymatic activity. Net fluorescence was calculated by subtraction of basal fluorescence in the absence of the enzyme. For each assay, the IC_{50} was calculated using a linear regression on the linear part of the sigmoid curve.

Lipid peroxidation assay

For determination of the ability to inhibit iron-dependent lipid peroxidation, membrane brain homogenate from rats was exposed to Fe^{2+} and ascorbic acid. The resulting lipid peroxidation was evaluated by the formation of malonaldehyde (MDA) (Esterbauer and Cheeseman 1990), the main decomposition product of peroxides derived from polyunsaturated fatty acids, using the chromogenic reagent *N*-methyl-2-phenylindole, which reacts with MDA to yield a stable chromophore with maximal absorbance at a wavelength of 586 nm. Microsomes were prepared from cerebral cortices removed from anaesthetized Sprague–Dawley rats. The cortices were rinsed immediately in ice-cold 20 mM Tris-HCl, pH 7.4. Tissues were homogenized in the same buffer and centrifuged for 15 min at 515 *g* at 4°C. Pellets were discarded and supernatants aliquoted in Beckmann centrifugation tubes (volume equivalent to 0.5 g fresh tissue). Tubes were then centrifuged for 20 min at 50 000 *g* at 4°C. Supernatants were discarded and pellets stored at −80°C pending lipid peroxidation assays. The procedure used for this study is the 96-well formatted protocol of Esterbauer *et al.* (1991). Adaptation to a multiwell format was performed in our laboratory. Five microlitres of diluted antioxidant or vehicle were transferred in a 1-mL deep-well 96-well plate (in duplicate). Frozen microsomes were resuspended in 20 mM Tris-HCl, pH 7.4, using 1 mL per 0.1 g tissue. A 40-μL aliquot of suspension was then transferred into the deep-well reaction plate. Plates were incubated at 37°C for 15 min with agitation. A peroxidation mixture was prepared by mixing together equivalent volumes of distilled water, 40 mM EDTA, 160 mM ascorbate and 40 mM $FeCl_2$. Five microlitres of this mixture was dispensed into each well of the reaction plate except for the control wells (non-peroxidized controls), which gave final concentrations of 1 mM EDTA and $FeCl_2$, and 4 mM ascorbate. Plates were kept for 30 min at 37°C with agitation to allow lipid peroxidation, then 160 μL chromogenic reagent (10 mM *N*-methyl-2-phenylindole in 100% acetonitrile) was added, followed 10 s later by 40 μL 37% HCl. Visualization of MDA formed was obtained after incubation at 45°C for 45 min. The separation of membranes and supernatant was obtained after vacuum filtration on a 96-well membrane screen MANALY50 (Millipore, Guyancourt, France). The MDA content was measured as the absorbance at 586 nm with a microplate reader (iEMS Reader MF, Labsystems, Les Ulis, France). The results were expressed as IC_{50} values.

Cell culture

Human neuroblastoma (SHSY5Y) cells and C6 glioma cells were obtained from the American Type Culture Collection (Manassas, Virginia, USA). Cells were maintained in DMEM supplemented with 10% FCS in a flask. The day before the experiment, cells were

EXHIBIT A

grown on 96-well plates at 50 000 and 25 000 cells per well respectively, at 37°C and 5% CO_2 in a humidified atmosphere in DMEM supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin.

Calpain *in situ* assay

On the day of the experiment, cells were either washed three times with serum-free DMEM and 40 mM HEPES or kept in medium containing 10% FCS, as for cell protection assays. Cells were pretreated for 1 h with calpain inhibitor in DMEM in the presence or absence of 10% FCS. The enzyme substrate (Suc-Leu-Tyr-AMC) and MTX were then added for testing the inhibitory effect of compounds on the intracellular calpain activation induced by the toxin. Fluorescence was read using a fluorescent plate-reading system (Victor Wallac) at 380 nm excitation and 460 nm emission. To determine the capacity of compounds to inhibit the intracellular calpain, the difference in fluorescence between the MTX-treated and -untreated control wells, corresponding to 100% intracellular calpain activation, was determined and the IC_{50} was estimated using a linear regression on the linear part of the sigmoid curve.

8-*Iso*prostane *in situ* assay

8-*Iso* PGF2α is an isoprostane produced by the non-enzymatic peroxidation of arachidonic acid in membrane phospholipids (Morrow *et al.* 1998). F2-isoprostanes (F2-IPs) are formed by the free radical-catalysed oxidation of arachidonic acid. The measurement of F2-IPs, especially 8-epi-PGF2α, is recognized as a reliable marker of lipid peroxidation and is currently used as a sensitive index of oxidative stress *in vivo*. (Gopaul *et al.* 2000). On the day of the experiment, cells were pretreated for 1 h with the antioxidant compound and MTX was then added. After 3 h, the supernatant were frozen at –20°C. The level of 8-*iso* PGF2α was measured by EIA kit. The results were expressed as IC_{50} values.

Cell viability measurement

A colorimetric assay that uses cell proliferation reagent WST-1 was employed for the quantification of cell viability, based on the cleavage of the tetrazolium salt WST-1 (slightly red) by mitochondrial dehydrogenases in viable cells to formazan (dark red). An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the well. This augmentation in enzyme activity leads to an increase in the amount of formazan dye formed, which is directly correlated with the number of metabolically active cells in the culture. The formazan dye produced by metabolically active cells was quantified by a scanning multiwell spectrophotometer by measuring the absorbance of the dye solution at 420–480 nm with a reference wavelength > 600 nm. On the day of the experiment, cells were pretreated for 1 h with 50 µL compound 1 plus 50 µL vehicle or 50 µL compound 2 per well, and MTX was then added. To determine 100% viability, control wells without addition of MTX were prepared. The difference in optical density between the control wells and the MTX treated cells corresponds to the no-survival fraction (NSF) after treatment with toxin and represents 100% cell death. From the calculated NSF obtained after treatment with compound and MTX, the percentage protection against cell death induced by MTX was determined. The effective concentration that protected 50% of C6 cells (EC_{50}) was calculated on the linear part of the sigmoid curve. Cell protection was confirmed by examination of cells by phase-contrast microscopy (data not shown). In the text, the percentages of cell protection are expressed as the mean +/– SEM.

Theoretical association

In any *in vitro* investigation of drug combinations, it is important to define the drug interactions in a quantitative manner. An excellent review on the *in vitro* quantification of drug interactions was published by Valeriote and Lin (1975). Briefly, with anticancer drugs that induced cell death, the survival fraction (SF) of each anticancer drug individually (SF_A or SF_B) and the SF of the anticancer drug combination (SF_{A+B}) were determined experimentally. If SF_{A+B} was less than the product of the survival fraction of each individual drug ($SF_A \times SF_B$), the ratio of SF_{A+B} to ($SF_A \times SF_B$) was below 1 and the anticancer drug interaction was defined as synergistic.

In this study, we adapted this methodology to quantify the interactions between compounds. With compounds that induced cell protection, the NSF of each protective drug individually (NSF_A or NSF_B) and the NSF of the protective drug combination (NSF_{A+B}) was determined experimentally. If NSF_{A+B} was less than the product of each individual drug ($NSF_A \times NSF_B$), the ratio of NSF_{A+B} to ($NSF_A \times NSF_B$) was below 1 and the protective drug interaction was defined as synergistic. Results were expressed as the percentage protection against cell death induced by MTX. Thus, if the ratio of observed protection to theoretical protection was greater than 1, the protective drug interaction was defined as synergistic; if

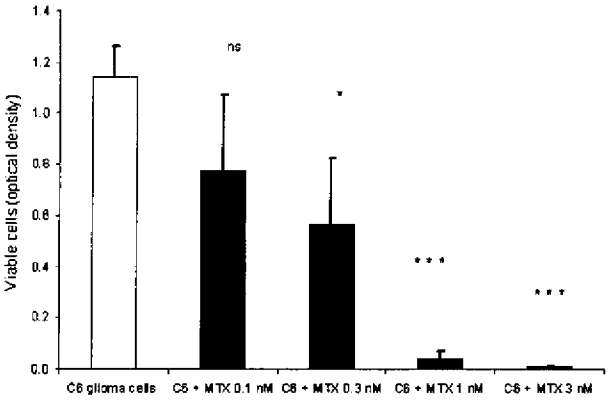


Fig. 2 Effect of MTX on C6 glioma cells: concentration dependence of MTX-induced decrease of viable cells. Cells were seeded at a density of 25 000 cells/100 µL per well of a 96-well microtitre plate the day before the experiment and treated with various concentrations of MTX (0.1, 0.3, and 1 nM). A colorimetric assay for the quantification of cell viability, based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells, was used. The formazan formed by the cleavage of tetrazolium salt was monitored using a ELISA plate-reading system at 420–480 nm with a reference wavelength above 600 nm. The amount of formazan dye formed was directly related to the number of metabolically active cells in the culture and expressed as optical density. Values are mean ± SEM ($n = 7$). Statistical analysis was by Student *t*-test. ns, Not significant; *$0.05 < p < 0.01$; ***$p < 0.001$ versus control C6 glioma cells.

equal to or less than 1, the interaction was defined as additive or antagonistic respectively.

Results

Effect of MTX on C6 glioma cells

The first step of this study was to establish the C6 cell culture conditions and cell viability in the presence or absence of various concentrations of MTX (Fig. 2). As expected, MTX induced, in a concentration–dependent manner, a loss of viable C6 cells that was quantified by the amount of formazan dye formed. Cell viability after treatment with MTX at 0.1 nM represented 68% of control values; however, this loss of 32% of viable cells was not significant by *t*-test analysis. At 0.3, 1 and 3 nM, MTX significantly decreased the number of C6 cells with a loss of 51, 96 and 98% of viable cells respectively. Cell mortality induced by MTX reached a plateau from 1 nM MTX.

The second step was to determine whether intracellular calpain activation was induced by MTX in C6 glioma cells under our conditions. The intracellular calpain activation assay was carried out in the presence or absence of several concentrations of MTX (Fig. 3). Calpain activation

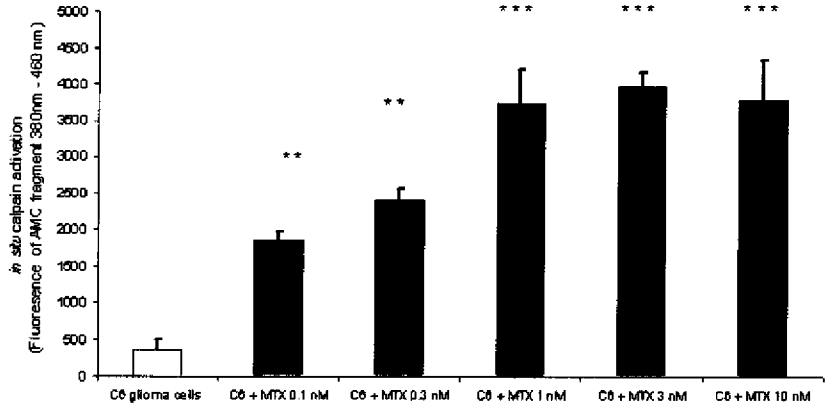


Fig. 3 Effect of MTX on C6 glioma cells: concentration dependence of MTX-induced intracellular calpain activation. Cells were seeded at a density of 25 000 cells/100 μL per well of a 96-well microtitre plate the day before the experiment and treated with MTX. The substrate Suc-Leu-Tyr-AMC, which can cross intact cell membranes, was added at 0.5 mM/10 μL. The fluorescence of the cleavage product, AMC, liberated from the peptide, was monitored using a fluorescent plate-reading system with filter settings fluorescence excitation 380 nm and emission 460 nm. To determine the background, control wells without addition of MTX were prepared. The amount of AMC fragment formed was directly related to the calpain activity in the cell culture. Values are mean ± SEM ($n = 3$). Statistical analysis was by Student *t*-test. **0.01 < p < 0.001; ***p <0.001 versus control C6 glioma cells.

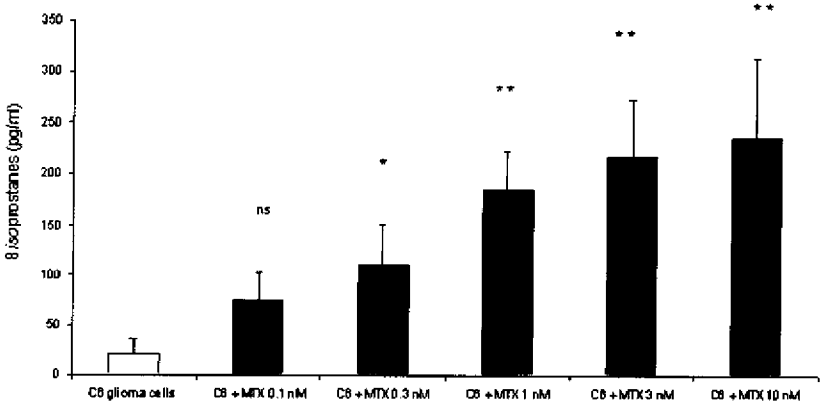


Fig. 4 Effect of MTX on C6 glioma cells: concentration dependence of MTX-induced intracellular 8-*iso*prostane production. The *iso*prostane 8-*iso* PGF2α is produced by the non-enzymatic peroxidation of arachidonic acid in membrane phospholipids. Cells were seeded at a density of 25 000 cells/100 μL per well of a 96-well microtitre plate the day before the experiment and treated with various concentrations of MTX. The level of 8-*iso*prostane was measured using an EIA kit. Values are mean ± SEM ($n = 5$). Statistical analysis was by Student *t*-test. ns, not significant; *0.05 < p < 0.01; **0.01 < p < 0.001 versus control C6 glioma cells.

EXHIBIT A

Antioxidant	Inhibition of lipid peroxidation induced by Fe^{2+} in rat brain microsomes IC_{50} (nM)	Inhibition of 8-*iso*prostane release in C6 glioma cells IC_{50} (μM)
2-Methoxy-10H-phenothiazine	233 ± 78	0.36 ± 0.03
4-Hydroxydiphenylamine	99 ± 27	1.92 ± 0.28
BHT	4320 ± 580	101.3 ± 24.44

MDA is one product that results from lipid peroxidation. The MDA content was measured by the absorbance at 586 nm. For 8-*iso* PGF2α determination, cells were seeded at a density of 25 000 cells/100 μL per well of a 96-well microtitre plate, pretreated for 1 h with antioxidant at concentrations between 0.1 and 100 μM, and then treated with MTX at 1 nM. The levels of 8-*iso* PGF2α in the supernatant were measured using an EIA kit after 3 h. The results are expressed as mean ± SEM IC_{50} values ($n = 3$).

Table 1 Inhibition of lipid peroxidation induced by Fe^{2+} in rat brain microsomes and of *8-iso* PGF2α release produced by the non-enzymatic peroxidation of arachidonic acid in membrane phospholipids in C6 glioma cells

occurred in a concentration-dependent manner after addition of MTX at 0.1–10 nM. Significant calpain activation was observed at each concentration of MTX tested. Maximal intracellular calpain activation was reached at 1 nM.

The third step was to determine whether MTX was able to increase lipid peroxidation, as measured by 8-*iso*prostane formation, under our conditions (Fig. 4). The 8-*iso*prostane level was increased dose dependently after addition of MTX at 0.1–10 nM. The specific product of non-enzymatic lipid peroxidation was increased three-fold after treatment with MTX at 0.1 nM, but this increase was not significant by *t*-test analysis. MTX at 0.3, 1, 3 and 10 nM significantly increased the level of 8-*iso* PGF2α by five-, nine-, 10- and 10-fold respectively; the maximal increase in 8-*iso* PGF2α level was reached from 1 nM.

An MTX concentration of 1 nM, which induced a significant level of cell death associated with a high level of calpain activation and lipid peroxidation, was used in further experiments.

Table 2 Inhibition of human calpain 1 enzyme and intracellular calpain activity by various calpain inhibitors

Calpain inhibitor	Inhibition of human calpain 1 IC_{50} (nM)	Inhibition of calpain in C6 glioma cells IC_{50} (μM) Without serum	With 10% serum
Z-Leu-Leu-H	6.7 ± 0.58	1.9 ± 0.41	26.4 ± 6.31
Z-Leu-Phe-H	9.14 ± 0.79	2.3 ± 0.27	19.8 ± 2.59
Calpeptin	7.41 ± 2.5	4.8 ± 2.62	67.4 ± 16.74
PD150606	5539 ± 841	38.0 ± 7.99	102.4 ± 23.13

Enzymatic inhibition was measured against isolated human calpain 1 and cellular inhibition of calpain activity was measured in C6 glioma cells. Cells were seeded at a density of 25 000 cells/100 μL per well of a 96-well microtitre plate the day before the experiment, pretreated with product at concentrations between 0.1 and 100 μM, and then treated with MTX at 1 nM. This assay was performed under two conditions, in the absence and presence of 10% FCS corresponding to the medium used in cell protection and 8-*iso*prostane assays. The substrate Suc-Leu-Tyr-AMC was added at 0.5 mM/10 μL. The fluorescence of the cleavage product AMC was monitored using a fluorescent plate-reading system (excitation at 380 nm and emission at 460 nm). The amount of AMC fragment formed was directly related to the calpain activity. The difference in net fluorescence between MTX-treated and -untreated cells incubated with vehicle was measured. This difference in fluorescence represented the 100% calpain activity induced by MTX. For each concentration of the tested compound, the percentage *in situ* calpain activity inhibition was calculated. For each drug, the IC_{50} was calculated using a linear regression on the linear part of the sigmoid curve. Values are mean ± SEM.

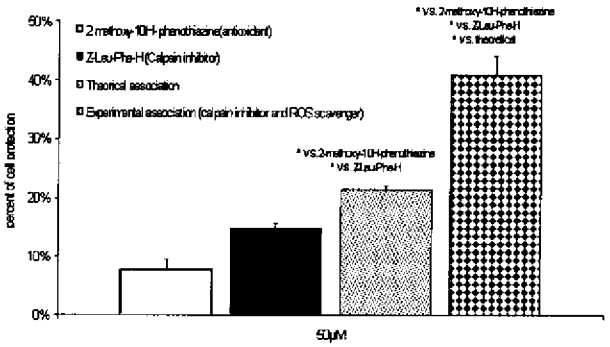


Fig. 5 Effect of the antioxidant 2-methoxy-10H-phenothiazine and the calpain inhibitor Z-Leu-Phe-H, and their combination, on MTX-induced C6 glioma cell death. Cells were seeded at a density of 25 000 cells/100 μL per well of a 96-well microtitre plate. The day after, cells were pretreated for 1 h with antioxidant (A), calpain inhibitor (B) or both compounds, followed by the addition of 1 nM MTX. To determine 100% viability, control wells without addition of MTX were prepared. The difference in optical density between MTX-treated and -untreated cells incubated with vehicle was evaluated. This difference represents the 100% NSF induced by MTX. The NSF of each protective drug individually (NSF_A or NSF_B) and that of the drug combination (NSF_{A+B}) were determined experimentally. If the NSF_{A+B} was lower than the product of each individual drug ($NSF_A \times NSF_B$), the protective drug interaction was defined as synergistic. Results are presented as the percentage cell protection, and the protective drug interaction was defined as synergistic when the observed protection was higher than the theoretical protection. Values are mean ± SEM ($n = 3$). *$p < 0.05$ (Student *t*-test).

EXHIBIT A

Effects of calpain inhibitors and antioxidants

Several antioxidants were tested for their ability to inhibit lipid peroxidation in acellular assays and their capacity to inhibit the 8-*iso*prostane release induced by MTX in C6 glioma assays (Table 1). The results of acellular assays showed that two of the three antioxidants tested, 2-methoxy-10H-phenothiazine and 4-hydroxydiphenylamine, were potent antioxidants and were more potent than BHT in inhibiting the lipid peroxidation induced by Fe^{2+} in rat brain microsomes. In a C6 glioma cell assay, the inhibition of F2-IP release induced by MTX was observed with the three agents, indicating that they were all cell-permeable antioxidants. The IC_{50} calculated in cell culture conditions, however, was higher than that in acellular assays, especially for 4-hydroxydiphenylamine and BHT (Table 1). Under these conditions, 2-methoxy-10H-phenothiazine seemed to be the most potent cell-permeable antioxidant of the three compounds tested.

In parallel, several calpain inhibitors were tested to determine their inhibitory potencies either in human calpain 1 enzyme (acellular) or intracellular calpain activation (induced by MTX) assays. Z-Leu-Leu-H, calpeptin and Z-Leu-Phe-H were much more potent inhibitors of the isolated enzyme than PD150606. The inhibitory effect of these compounds on intracellular calpain activation induced by MTX in C6 glioma cells is summarized in Table 2. This assay was performed in the absence and presence of FCS to reproduce the experimental conditions used in cell protection and 8-*iso*prostane assays. In the absence of FCS, the same ranking order of calpain inhibitors was observed between

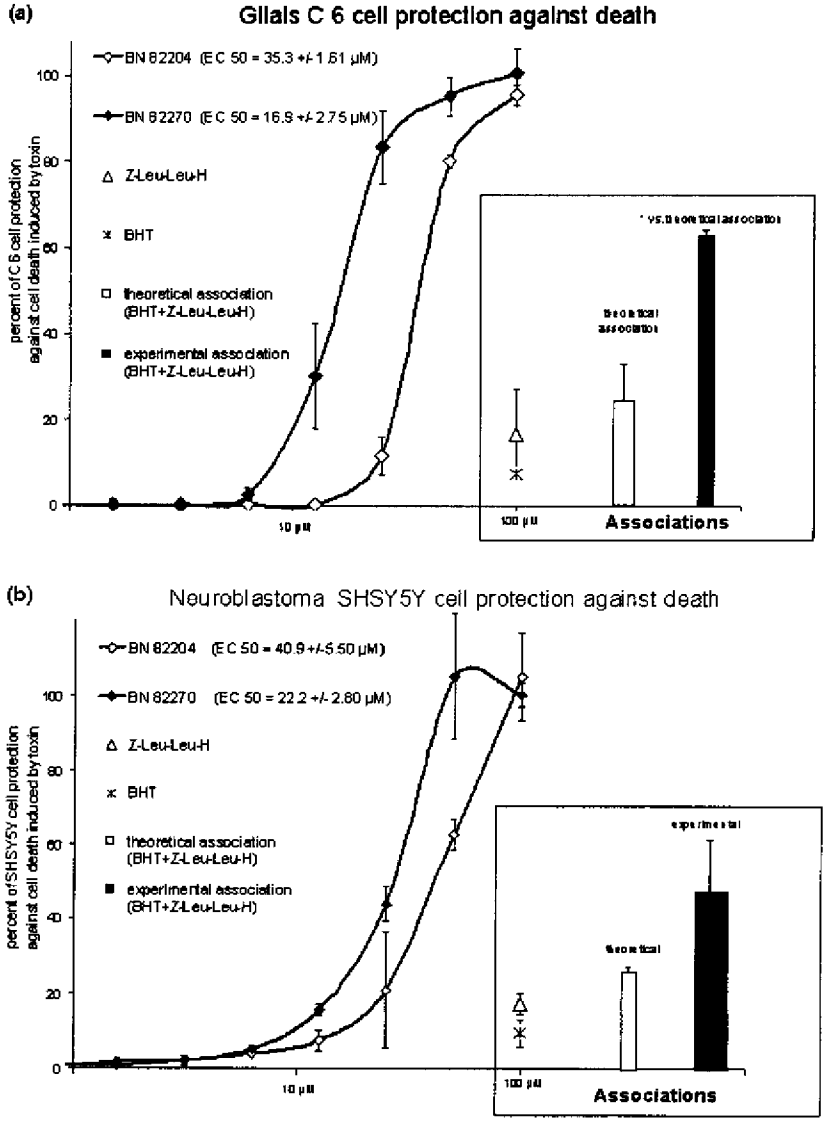


Fig. 6 Effects of BN 82204, its pro-drug BN 82270, BHT, Z-leu-Leu-H and their combination on MTX-induced cell death in C6 glioma cells (a) and neuroblastoma SHSY5Y cells (b). Cells were seeded at a density of 25 000 cells (a) *and* 50 000 cells (b) per 100 µl per well of a 96-well microtitre plate. The day after, cells were pretreated for 1 h with synthetic molecules, antioxidant (A), calpain inhibitor (B) and both compounds followed by addition of 1 nM MTX. To determine 100% viability, control wells without addition of MTX were prepared. The difference in optical density between MTX-treated and -untreated cells incubated with vehicle was measured. The difference in optical density represents the 100% NSF induced by MTX. In this series of experiments, MTX induced 91.7 ± 0.24% C6 cell loss and 93.2 ± 2.44% SHSY5Y cell loss. The NSF of each protective drug individually (NSF_A or NSF_B) and the NSF of the drug combination (NSF_{A+B}) were determined experimentally. If NSF_{A+B} was lower than the product of each individual drug ($NSF_a \times NSF_b$), the protective drug interaction was defined as synergistic. Results are presented as the percentage cell protection, and the protective drug interaction was defined as synergistic when the observed protection was higher than the theoretical protection. EC_{50} values were estimated on the linear part of the sigmoid curve. Values are mean ± SEM ($n = 3$). Statistical analysis was by Student *t*-test on the percentage of cell protection, $^{**}0.01 < p < 0.001$.

EXHIBIT A

Table 3 Selectivity of BN 82204 for proteases

	IC_{50} (nM)					
	Cysteine proteases			Aspartyl protease	Serine protease	Rabbit 20S proteasome
	Human calpain 1	Porcine calpain 2	Human cathepsin L	Human caspase 3	Bovine α-chymotrypsin	
BN 82204	43 ± 8.04	52.6 ± 9.05	38.7 ± 11.67	Not active	Not active	Not active
Benchmarks	Z-Leu-Leu-H	Z-Leu-Leu-H	Cathepsin L Inh. IV	Caspase 3 Inh. I	α-Chymotrypsin Inh. I	MG132
	9.9 ± 2.10	8.0 ± 0.47	9.3 ± 3.37	4.3 ± 0.54	58.6 ± 10.48	6.2 ± 2.91

Enzymatic inhibition was measured against isolated cysteine proteases (calpain 1–2, cathepsin L), aspartyl protease (caspase 3), serine protease (α-chymotrypsin) and proteasome activity. All assays were based on the ability of the enzymes to cleave synthetic peptidic fluorescent substrates: Suc-Leu-Tyr-AMC for human calpain 1 and porcine calpain 2, Cbz-Phe-Arg-AMC for human cathepsin L, AC-DEDV-AMC for human caspase 3, Suc-Ala-Ala-Pro-Phe-AMC for bovine α-chymotrypsin, and Suc-Leu-Leu-Val-Tyr-AMC for rabbit 20S proteasome. The fluorescence of the cleavage product, AMC, was monitored using a fluorescent plate-reading system (excitation at 380 nm and emission at 460 nm). The amount of AMC fragment formed was directly related to the isolated enzymatic activity. For each assay, the IC_{50} was calculated using a linear regression on the linear part of the sigmoid curve. Values are mean ± SEM. Inh, inhibitor.

Table 4 Intracellular calpain inhibition of BN 82204 and its pro-drug BN 82270

	Inhibition of calpain 1–2 activity in C6 glioma cells IC 50 (μM)	
	Without serum	With 10% serum
BN 82204 (active principle)	31.1 ± 2.33	60.4 ± 8.79
BN 82270 (pro-drug)	10.9 ± 1.87***	26.6 ± 2.24*

In C6 glioma cells, inhibition of calpain activity was measured by inhibition of specific substrate cleavage. Cells were seeded at a density of 25 000 cells/100 μL per well of a 96-well microtitre plate the day before the experiment, pretreated with product at concentrations between 0.1 and 100 μM, and then treated with MTX at 1 nM. The assay was performed under two conditions, in the absence and presence of 10% FCS. The substrate Suc-Leu-Tyr-AMC was added at 0.5 mM/10 μL. The fluorescence of the cleavage product AMC was monitored using a fluorescent plate-reading system (excitation at 380 nm and emission at 460 nm). The amount of AMC fragment formed was directly related to the calpain activity in the cell culture. The difference in net fluorescence between MTX-treated and -untreated cells incubated with vehicle was measured. The difference in fluorescence represents the 100% calpain activity induced by MTX. For each concentration of tested compound, the percentage inhibition of calpain activity was calculated. For each drug, the IC_{50} was calculated using a linear regression on the linear part of the sigmoid curve. Values are mean ± SEM ($n = 3$). *$p = 0.05 < p < 0.01$, **$p = 0.01 < p < 0.001$ versus BN 82204 (Student *t*-test).

isolated enzyme and intracellular C6 glioma cell assays. Z-Leu-Leu-H, Z-Leu-Phe-H and calpeptin were shown to be more potent calpain inhibitors than PD150606. With 10% FCS in the cell culture, however, the IC_{50} values were much higher than those obtained without FCS for all compounds (Table 2). In addition, the classification was changed. In the presence of serum, Z-Leu-Leu-H and Z-Leu-Phe-H were between two and three times more potent than calpeptin in inhibiting intracellular calpain (Table 2). As in cultures lacking FCS, PD150606 was the least potent calpain inhibitor in the presence of FCS (Table 2). Either in the absence or presence of FCS, all calpain inhibitors were able to inhibit the intracellular activated calpain, indicating that they were all cell-permeable inhibitors. Of the four compounds tested, Z-Leu-Phe-H seemed to be the most effective cell-permeable calpain inhibitor, followed by Z-Leu-Leu-H.

Effects of calpain inhibitors, antioxidants and their combination in cell protection

We used the best cell-permeable and potent calpain inhibitor (Z-Leu-Phe-H) and antioxidant (2-methoxy-10H-phenothiazine), determined as described previously, to examine their effects individually and in combination on cell protection. 2-Methoxy-10H-phenothiazine or Z-Leu-Phe-H used alone had only a marginal protective effect (less than 20%) on cell death induced by MTX (Fig. 5). However, the percentage protection obtained with the combination of both compounds at 50 μM was significant ($p < 0.05$) and higher than the theoretical percentage protection. The calculated ratio of observed to theoretical protection was 1.91. As described in materials and methods, a ratio above 1 suggested that this combination induces a synergistic protection against cell death (Fig. 5).

Several other combinations of antioxidants and calpain inhibitors such as BHT–PD150606, BHT++calpeptin, 2-methoxy-10H-phenothiazine+calpeptin, 4-hydroxydiphenylamine++Z-Leu-Leu-H and BHT–Z-Leu-Leu-H were used to confirm the previous result. Each compound used alone had only a marginal protective effect (as illustrated in the insert in Fig. 6a for BHT+Z-Leu-Leu-H) and in the same range of efficacy. For all combinations, the calculated ratio of

EXHIBIT A

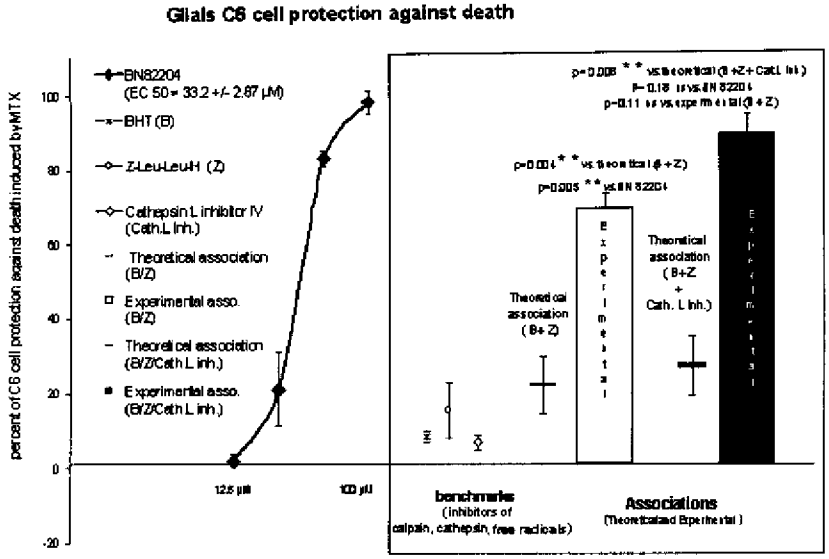


Fig. 7 Effect of BN 82270, BHT, Z-leu-Leu-H, cathepsin L inhibitor IV (Cath. L Inh.) and their combination on MTX-induced cell death in C6 glioma cells. Cells were seeded at a density of 25 000 cells/100 µL per well of a 96-well microtitre plate. The day after, cells were pretreated for 1 h with synthetic molecules, antioxidant (A), calpain inhibitor (B), cathepsin inhibitor (C) and combinations AB or ABC followed by addition of 1 nM MTX. To determine 100% viability, control wells without addition of MTX were prepared. The difference in optical density between MTX-treated and -untreated cells incubated with vehicle was measured. The difference in optical density represents the 100% NSF induced by MTX. In these series of experiments, MTX induced 96.26 ± 0.90% C6 cell loss. The NSF of each protective drug individually (NSF_A, NSF_B or NSF_C) and the NSF of the drug combination (NSF_{A+B} or NSF_{A+B+C}) were determined experimentally. If NSF_{A+B} or NSF_{A+B+C} was lower than the product of each individual drug [($NSF_A \times NSF_B$) or ($NSF_A \times NSF_B \times NSF_C$)], the protective drug interaction was defined as synergistic. Results are presented as the percentage cell protection, and the protective drug interaction was defined as synergistic when the observed protection was higher than the theoretical protection. EC_{50} values were estimated on the linear part of the sigmoid curve. Values are mean ± SEM ($n = 3$). Statistical analysis was by Student *t*-test on the percentage of cell protection, $^{**}0.01 < p < 0.001$.

observed to theoretical protection was above 1 (1.96, 2.85, 2.81, 2.25 and 2.70 respectively). These results showed that combination of an antioxidant and a calpain inhibitor led to a higher level of protection against cell death than was expected from the theoretical association between the compounds (as illustrated in Fig. 6a, insert). For all the combinations, the cell protection was synergistic but never exceeded 66%. It was found that this synergistic effect on cell protection was not associated with a synergistic effect of an antioxidant combined with a calpain inhibitor on MTX-induced calpain activation or lipid peroxidation. Indeed, we established that the combinations tested led to the same percentage inhibition of either calpain or 8-*iso*prostane levels as obtained with calpain inhibitors or antioxidants used alone (data not shown).

The effect of a combination of BHT and Z-Leu-Leu-H was also evaluated in neuroblastoma SHSY5Y cells. Calpain inhibitor or antioxidant used alone had only a marginal protective effect (less than 20%) (Fig. 6b, insert). As in the rat C6 glioma model, the combination of calpain and free radical inhibitor had a synergistic effect, with a calculated experimental to theoretical protection ratio of 1.73 (Fig. 6b, insert). Results obtained from the calpain inhibitor Z-Leu-Leu-H with free radical inhibitor BHT association showed the same range of cell protection in both cell lines (60.7 ± 3.28% in C6 cells and 44.1 ± 17% in SHSY5Y; $p = 0.29$). However, it was noted that the variability was higher in SHSY5Y cells than in glioma C6 cells (Figs 6a and b).

Characterization and effects of synthetic dual inhibitor BN 82204 and its pro-drug BN 82270 on calpain activity and lipid peroxidation

To reproduce the biological effects of a combination, BN 82204 was designed with dual activity, inhibition of both calpain and lipid peroxidation (Auvin *et al.* 2004). To increase cell permeability and therefore potentially its activity, an acetylated pro-drug, BN82270, was also synthesized, as described by Auvin *et al.* (2004). Biological characterization of the synthetic compound is reported in Table 3 for acellular assays and in Table 4 for cellular inhibition. Briefly, the enzymatic profile showed that BN 82204 inhibited calpain 1 and 2 but had no activity against proteasome, aspartyl protease (caspase 3) and serine

EXHIBIT A

protease (α-chymotrypsin) (Table 3). Futher investigation of the thiol protease family showed that BN 82204 was also a cathepsin L inhibitor and as such should be considered as a cysteine protease inhibitor. As expected, in an acellular system the pro-drug BN 82270 was devoid of effect on proteases owing to the acetyl group, which masks the cysteine protease pharmacophore. Antioxidant properties, measured by inhibition of lipid peroxidation in rat brain microsomes, were in the same range of activity for BN 82204 and BN 82270, which possess the same antioxidant pharmacophore (IC_{50} 173 ± 40 and 297 ± 70 nM respectively; $p = 0.16$).

In glioma C6 cells, cell permeability was shown with BN 82204, which decreased the 8-*iso*prostane level (IC_{50} 19.0 ± 0.68 μM) and inhibited intracellular calpain activity (IC_{50} 31.1 ± 2.33 μM in the absence and 60.4 ± 8.79 μM in the presence of serum) (Table 4). The pro-drug BN 82270 was significantly more potent in inhibiting the intracellular calpain activation induced by MTX than BN 82204 [IC_{50} 10.9 ± 1.87 μM in the absence of serum ($p = 0.0005$); 26.6 ± 2.24 μM in the presence of serum ($p = 0.01$)] (Table 4). As already observed with other calpain inhibitors (Table 2), the presence of 10% FCS increased the IC_{50} values.

Effects of BN 82204 and BN 82270 on cell protection

BN 82204 and BN 82270 were tested in cell protection assays. BN 82204 was a potent C6 cell or SHSY5Y cell protector *in vitro* with an EC_{50} of 35.3 ± 1.6 and 40.9 ± 5.50 μM respectively (Figs 6a and 6b). BN 82270 however, was significantly more effective than its parent compound BN 82204 in C6 and SHSY5Y cells [EC_{50} 16.9 ± 2.75 μM ($p = 0.0045$) and 22.2 ± 2.80 μM ($p = 0.0013$) respectively] (Figs 6a and 6b). Both compounds protected totally at 100 μM.

Supplementary experiments were carried out to elucidate the discrepancy between the complete protection obtained with BN compounds and the partial protection observed with the combinations, which never exceeded 66%. Because the enzymatic profile of BN 82204 showed cathepsin inhibition, a combination including a cathepsin L inhibitor was evaluated. In this new experiment, the cell protection due to Z-Leu-Leu-H+BHT (66.4 ± 6.44%) was significantly lower than that obtained with BN 82204 (97.6 ± 3.08% at 100 μM) ($p = 0.005$) (Fig. 7). Unexpectedly, the addition of a cathepsin L inhibitor to the combination of Z-Leu-Leu-H and BHT increased the level of cell protection (87.4 ± 7.05%), close to the total cell protection obtained with BN 82204 ($p = 0.18$) (Fig. 7).

Discussion

Calcium-dependent cytosolic calpains have been considered as promising therapeutic targets. Calpains seem to be implicated in various degenerative disorders including cerebral ischaemia, Parkinson's disease, myocardial ischaemia and trauma brain injury (Wang and Yuen 1994). The unifying features of these pathological conditions are that calcium serves as a trigger for cellular injury and may represent a crucial mediator of the degenerative response. It is thought that these enzymes primarily degrade cytoskeletal and nuclear scaffold proteins, which leads to cell death and consequent tissue degradation. In cell culture, activation of calpain can be achieved by MTX, a potent activator of changes in intracellular Ca^{2+} concentrations. MTX is a better calpain activator than the calcium ionophore A23187 (Wang *et al.* 1996). Konoki *et al.* (1999) demonstrated that MTX (0.3 nM) elicited a 10–20-fold increase in the level of Ca^{2+} influx in rat glioma C6 cells. Calpain activation after MTX treatment has been well established by Wang *et al.* (1996), who detected intracellular hydrolysis of a specific calpain substrate (Suc-Leu-Leu-Val-Tyr-AMC). Moreover, they showed that calpain inhibitors (calpain inhibitor I, MDL28170 and PD150606) inhibited hydrolysis of this specific substrate in MTX-treated SHSY5Y cells.

In our study, MTX also induced calpain activation associated with cell death in C6 glioma cells. Furthermore, a concentration-dependent increase in ROS was observed

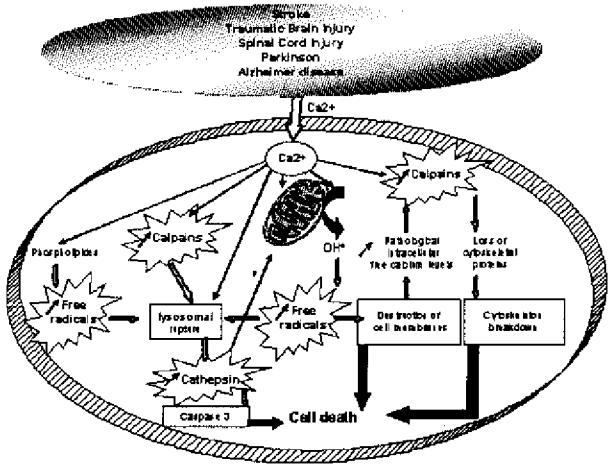


Fig. 8 Schematic diagram showing potential involvement of calpain, cathepsin and free radicals in cell death in neurological diseases. In the cascade of events leading to cell death, calpain is activated and free radicals are overproduced. Activation of calpain provokes degradation of several proteins such as cytoskeletal proteins (neurofilaments, microtubule-assocated protein 2 (MAP2) in dendrites, spectrin and tau in axons) which induces cytoskeletal breakdown. In parallel, from the phospholipids of the membrane or via the mitochondria, the overproduction of free radicals is followed by lipid peroxidation and destruction of the cell membrane, which at the end could also participate in increasing the level of calcium influx into the cells. Lysosomal rupture induces release of cathepsin which may trigger mitochondrial dysfunction, cleavage and activation of caspase 3. All these events (cytoskeletal breakdown, destruction of the cell membrane and lysosomal rupture) are implicated in cell death.

following MTX treatment. Interestingly, cell death caused by MTX exposure was only partially blocked by calpain inhibitors or antioxidants, suggesting that inhibition of one pathway is not sufficient to completely protect against cell death. Thus, inhibition of the two pathways (cytoskeleton breakdown and destruction of the cell membrane; Fig. 8) could be a neuroprotective strategy. The effect of a combination of a calpain inhibitor and an antioxidant was investigated. The present results clearly showed that such a combination was significantly protective and synergistic. Effectively, concomitant inhibition of the two pathways implicated in cell death events afforded greater cell protection than that expected from the theoretical association. These findings suggest that this approach might have promise in the treatment of degenerative disorders in which cell death is an important feature.

New compounds were therefore designed with dual activity (calpain inhibitor and antioxidant; Fig. 1). This strategy of combining two types of biological activity in a single chemical entity has already been performed successfully in our laboratory, with BN 80933, a dual inhibitor of nitric oxide synthase and free radicals, giving a potent anti-ischaemic agent (Chabrier *et al.* 1999). The concept of multifunctional molecules targeting different pathological pathways may be particularly well adapted to diseases involving necrotic cell death. In this context we found that BN 82204 totally protected C6 glioma cells, in contrast to the combination of a calpain inhibitor and an antioxidant, which produced a maximum cell protection of 66% whatever the co-treatment used.

The total efficacy of BN 82204 in the cell protection assay may be explained by its additional inhibitory effect on cathepsin. Indeed, cathepsins as well as calpains are thought to play a significant role in neuronal death such as that observed in ischaemia, Alzheimer's disease or in age-related neurodegeneration (Nakanishi 2003; Yamashima 2004). Furthermore, the beneficial effect obtained with BN 82204 was also improved by use of its pro-drug. BN 82270 carries an acetyl group to mask the calpain pharmacophore, and this improves the cell permeability (Auvin *et al.* 2004). The curves of cell protection and the calculated EC_{50} showed that treatment with the pro-drug was more effective than treatment with the parent compound.

In conclusion, inhibition of calpain activation and free radical overproduction pathways by two separate molecules used in combination affords synergistic protection against necrotic cell death of C6 cells and SHSY5Y cells. Furthermore, simultaneous inhibition of the calpain, cathepsin L and free radical pathways (Fig. 8) by BN 82204 and BN 82270 provides total protection in both neuronal cell types. These results suggest that use of multifunctional drugs might have some advantages over drug combinations and justify further investigations of BN 82270 in *in vivo* models of disorders involving necrosis.

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